APPLICATION OF BIOCHEMICAL SOLID-PHASE TECHNIQUES IN THE STUDY OF HORSE LIVER ALCOHOL DEHYDROGENASE

Chromatography on immobilized adenine nucleotides and some methods of studying subunit interactions in the enzyme

Lars Andersson

Lund 1978

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av

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Methods have been developed to purify horse liver alcohol dehydrogenase (HLADH) as well as separation of the isozymes by using general ligand affinity chromatography. The use of magnetic affinity adsorbents for purification of the enzyme and separation of the isozymes has also been demonstrated.

The interaction between HLADH and an immobilized NADH-analogue has been studied. It has been shown by using spectrofluorometry and affinity chromatography on low substituted affinity gels that the binding on solid-phase is similar to the binding in free solution.

Immobilized single subunits of HLADH have been prepared and hybrids have been formed between immobilized subunits of the SS isozyme and soluble subunits of the EE isozyme. Results based on the dissociation and reassociation of immobilized HLADH suggest that single subunits of HLADH are inactive and that dimer formation is a prerequisite for enzyme activity.

A method for active-site selective carboxymethylation on solid-phase of bioaffinity-bound HLADH has been demonstrated. The monoalkylated enzyme prepared in this way was half as active as native enzyme when using ethanol or benzaldehyde as substrates, indicating that both subunits in native HLADH may be kinetically equivalent.

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To my parents,
Ingun and Jonas

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I SUMMARY

Methods have been developed to purify and isolate horse liver alcohol dehydrogenase (HLADH) by general ligand affinity chromatography. The enzyme has been purified in a single step from a crude extract on an immobilized 5'-AMP-analogue and isozymes of the enzyme have been separated on the same immobilized general ligand. Preparative purification of the homogeneous steroid-active isozyme (SS) using chromatography on 5'-AMP-Sepharose has been demonstrated. The use of magnetic affinity adsorbents for purification of the enzyme and separation of the isozymes has been shown and it has been demonstrated that magnetic affinity adsorbents are a valuable tool for quick retrieval of the enzyme from crude extracts.

The interaction between HLADH and an immobilized NADH-analogue has been studied. It has been shown by using spectrofluorometry and affinity chromatography on low substituted affinity gels that the binding on solid-phase is similar to the binding in free solution.

Immobilized single subunits of HLADH have been prepared and hybrids have been formed between immobilized subunits of the SS isozyme and soluble subunits of the EE isozyme. Results based on the dissociation and reassociation of immobilized HLADH suggest that single subunits of HLADH are inactive and that dimer formation is a prerequisite for enzyme activity. Subunit exchange between immobilized subunits and soluble dimeric enzyme has also been suggested.

A method for selective carboxymethylation on solid-phase of bioaffinity-bound HLADH has been demonstrated. The monoalkylated enzyme prepared in this way was half as active as native enzyme when using ethanol or benzaldehyde as substrates. Steady-state kinetic results obtained with the monoalkylated HLADH are discussed in terms of enzyme action.

This thesis is based on the following papers:

I. Separation of Isozymes of Horse Liver Alcohol Dehydrogenase and Purification of the Enzyme by Affinity Chromatography on an Immobilized AMP-Analogue.
Andersson, L., Jörnvall, H., Åkeson, Å. and Mosbach, K. (1974) Biochim. Biophys. Acta 364, 1-8.

II. Preparative Purification of Homogeneous Steroid-Active Isozyme of Horse Liver Alcohol Dehydrogenase by Affinity Chromatography on an Immobilized AMP-Analog.
Andersson, L., Jörnvall, H. and Mosbach, K. (1975) Anal. Biochem. 69, 401-409.

III. Magnetic Ferrofluids for Preparation of Magnetic Polymers and Their Application in Affinity Chromatography.
Mosbach, K. and Andersson, L. (1977) Nature 270, 259-261.

IV. Biospecific Interactions on Solid-Phase between Alcohol Dehydrogenase and an Immobilized NADH-Analogue Studied by Fluorometry.
Andersson, L., Larsson, P.-O. and Mosbach, K. (1978) FEBS Lett. 88, 167-171.

V. The Use of Biochemical Solid-Phase Techniques in the Study of Alcohol Dehydrogenase.
I. Some Studies on Subunit Interactions in Alcohol Dehydrogenase with Subunits Covalently Bound to Agarose.
Andersson, L. and Mosbach, K. (1978). Submitted for publication.

VI. The use of Biochemical Solid-Phase Techniques in the Study of Alcohol Dehydrogenase.
II. Selective Carboxymethylation of Bioaffinity-Bound Alcohol Dehydrogenase.
Andersson, L. and Mosbach, K. (1978). Submitted for publication.

The papers will be referred to in the text by their Roman numerals.

II INTRODUCTION

The two interrelated fields immobilized enzymes and affinity chromatography have received widespread attention in recent years. Their main feature is the use or study of biological molecules while they are bound or immobilized on a solid-phase or support. Immobilized enzymes have, for instance, been successfully applied in the field of enzyme technology and also as analytical devices, such as enzyme electrodes and enzyme thermistors (1,2). Furthermore, immobilized enzymes have been used as a help in answering questions of a more fundamental biochemical character (2). Among the examples of the latter application are studies which concern multi-step enzymes (carried out to simulate enzyme organization in the living cell), subunit interactions in oligomeric enzymes and protein refolding. Affinity chromatography or bioaffinity chromatography, on the other hand, has become a widely accepted technique for the purification of enzymes and other biological macromolecules (3,4). The method exploits the unique biological properties of these molecules to bind ligands specifically and reversibly. The immobilized affinity ligand is usually a small molecule but it can also be a macromolecule, for instance, a protein or a nucleic acid.

Two major approaches of affinity chromatography are known: the specific ligand approach (5) and the general ligand or group-specific approach (6). In the former case, the specific affinity ligand will only recognize its complementary enzyme whereas in general ligand affinity chromatography a whole group of enzymes show affinity for the immobilized ligand. Examples of general ligands are adenine nucleotides which interact with several groups of enzymes, such as dehydrogenases, kinases and CoA-dependent enzymes, and concanavalin A which binds polysaccharides and glycoproteins.

Horse liver alcohol dehydrogenase (HLADH) is a NAD^+-dependent enzyme which has been extensively studied (7). The enzyme is dimeric (mol wt 84000) and contains 2 zinc ions per enzyme subunit. One of these zinc ions is required for

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Abbreviations used: HLADH = horse liver alcohol dehydrogenase; LDH = lactate dehydrogenase; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; MDH = malate dehydrogerase; BSA = bovine serum albumin; 5'-AMP-Sepharose = N^6-(6-aminohexyl)-5'-AMP substituted Sepharose 4B; NADH-Sepharose = N^6-[N-(6-aminohexyl)-carbamoylmethyl]-NADH substituted Sepharose 2B.

catalysis and the other may have a structural function. The complete amino acid sequence of the horse enzyme is known as well as its three-dimensional structure which has been determined to a resolution of 2.4 Å by X-ray crystallography. The mechanism of action of HLADH has also been investigated in great detail, but problems still remain especially concerning the role subunit interactions play in the enzyme action. Furthermore, although HLADH reversibly catalyzes the oxidation of a variety of alcohols by the coenzyme NAD^+, the physiological function of the liver enzyme is not fully understood.

The purpose of the present study has been to apply biochemical solid-phase techniques in the study of HLADH. Papers I-III describe methods to purify HLADH as well as to separate isozymes of the enzyme with general ligand affinity chromatography. Paper IV describes a spectrofluorometric method to study interactions between HLADH and an immobilized coenzyme-analogue using low substituted affinity gels. In this thesis the potential use of such affinity gels for binding studies of HLADH is also discussed. Papers V and VI concern methods used to study effects of subunit interactions in HLADH on the enzymic activity.

III GENERAL ASPECTS OF AFFINITY CHROMATOGRAPHY

In this part of the thesis some general principles relevant for all types of affinity chromatography will be discussed briefly. However, special reference will be given to general ligand affinity chromatography of enzymes on immobilized adenine nucleotides.

1. The matrix

The ideal matrix for affinity chromatography should form a loose and porous network to permit the free entry and exit of macromolecules throughout the entire matrix. It should be hydrophilic and not contain ionic groups which might cause nonspecific binding of macromolecules to the gel-matrix *per se* and, most important it must possess chemical groups which can be readily activated or modified to allow covalent coupling of the affinity ligand. In addition, the gel particles should be uniform, rigid and exhibit good flow properties. They should also be stable towards chemical and microbial degradation.

The most widely used support material for affinity chromatography is beaded agarose which has several properties of an ideal matrix. Agarose is a hydrophilic copolymer consisting of alternating residues of 3-linked β-D-galacto-

pyranose and 4-linked 3,6-anhydro-α-L-galactopyranose (8) that can easily undergo substitution reactions after activation, with for instance CNBr at high pH (9). On cross-linking with bifunctional reagents such as epichlorohydrin, divinylsulphone, or bisepoxides the agarose structure is also stabilized (10). Other examples of matrices that have been used as bed materials for affinity chromatography are cellulose (11), polyacrylamide gels (12), porous glass (13), dextran-coated ceramic beads (14) and iron oxide particles (15). Recently, copolymers of agarose and polyacrylamide, which have both amide and hydroxyl groups available for activation, have also proven of value as a matrix for affinity chromatography (16). Throughout this work beaded agarose (Sepharose; Pharmacia, Sweden) was used as support material for the affinity chromatography (I-IV,VI) and as matrix for the preparation of immobilized HLADH (V).

2. The ligand

The ligand to be immobilized, which in the case of adenine nucleotides can be a coenzyme (e.g. NAD(H), an inhibitor (e.g. 5'-AMP) or an effector molecule (e.g. c-AMP), must possess functional groups necessary for its coupling to a solid support. The ligand must be stable to withstand the conditions involved in chemical coupling, and also to experimental conditions used in the adsorption and elution of enzymes. Once the ligand has been immobilized it is important that it interacts both reversibly with the complementary protein and strongly so that satisfactory binding can be obtained on the matrix. Interactions in free solution corresponding to dissociation constants greater than 10^{-3}M are probably too weak to allow sufficient binding on solid-phase. These requirements are generally fulfilled by adenine nucleotides used in general ligand affinity chromatography.

2.1. The mode of attachment of the ligand

The ligand can be attached to the matrix either directly or, which is more common, via an extension arm or "spacer" that is inserted between the ligand and the matrix backbone to make the ligand more accessible for binding to the complementary protein (17). Attachment of the ligand to the spacer can be carried out in two principally different ways (Fig.1). The ligand is either coupled directly to a gel which has been already derivatized with a spacer carrying a functional group suitable for covalently attaching the ligand ("modular solid-phase approach") or it is first modified by substitution with a spacer in free solution and this spacer-ligand assembly is then covalently linked to the matrix through a functional group at the free end of the spacer

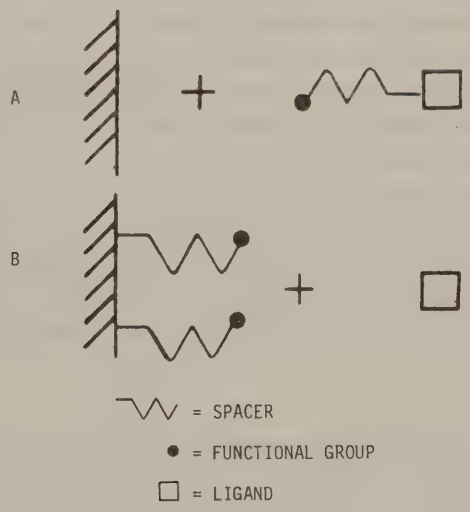

Fig.1. Two principal modes of attachment of a ligand to the matrix through a spacer. A. "Preassembled ligand approach". B. "Modular solid phase approach".

("preassembled ligand approach"). Although the former method is more convenient than the latter, which often requires rather elaborate organic chemistry, there is a big risk that affinity adsorbents prepared according to the modular solid-phase approach will contain unsubstituted extension arms which may interact in a nonspecific manner with the protein to be purified, and also since most affinity ligands have several potentially reactive sites a heterogeneous affinity adsorbent may be obtained. In contrast, coupling of a preassembled ligand to the matrix yields a chemically well-defined gel without any interfering unsubstituted extension arms. Furthermore, the synthesis of a preassembled ligand offers the advantage that the dissociation constant for binding of the ligand-analogue to the complementary protein in free solution can be determined. This is of great importance because the applicability of the immobilized ligand-analogue as an affinity adsorbent can be predicted prior to immobilization. In the present study only preassembled adenine nucleotide-analogues were used (I-IV, VI).

2.2. The point of attachment of the ligand

The affinity ligand must be covalently linked to the matrix via a part of its structure which is not essential for binding to its complementary protein, or with regards to immobilized coenzymes through a group that is important for its

function. For example, on adenine nucleotide coenzymes, such as NAD^+ and $NADP^+$ there are four different reactive positions which have been utilized for anchoring these compounds via a spacer to the matrix. These points of attachment include substitution on the N^6- and C8-positions of the adenine ring, substitution through the hydroxyls of the ribose (s) and substitution on the nicotinamide moiety. Another possible point of attachment on adenine mononucleotides, such as AMP, ADP, and ATP, is the terminal phosphate group. For a comprehensive review about the synthesis of various adenine nucleotide-analogues and their use in general ligand affinity chromatography see reference (6).

3. The effect of the extension arm

The effect on the ligand-enzyme interaction by attaching the ligand through an extension arm may not only be due to a decreased steric hindrance imposed by the matrix backbone, as mentioned earlier, but also to an increased flexibility and mobility of the immobilized ligand (18). It has also been suggested that the possible barrier to binding due to restricted diffusion across the ordered layer of water molecules surrounding the hydrophilic matrix may be diminished by coupling the ligand via a spacer (which is supposed to protrude out of this region of the solvent) (19). Optimal accessibility of the immobilized ligand is usually observed when the length of the extension arm is in the region of 8-10 Å which corresponds to a fully extended hydrocarbon chain containing 6-7 methylene groups (20). Beyond this spacer length the binding of the enzyme is not further increased but remains constant, however, it has also been reported to decline (19). For the latter observation, "curling-back" of the hydrophobic ligand onto the hydrophobic extension arm may be a possible explanation (21).

The chemical nature of the extension arm may in itself affect the chromatographic behaviour of enzymes on affinity columns. Since enzymes are polyelectrolytes and since they also may possess hydrophobic areas near their surface nonspecific binding due to ionic groups on the spacer and/or hydrophobic interaction with methylene groups comprising the spacer may be likely to occur (21,22). The enzyme may interact with the extension arm either when it is bioaffinity-bound resulting in a reinforced bioaffinity or so called "compound affinity" (23), or in a"true" nonspecific manner without any biospecific binding to the ligand. Nonspecific interaction of the latter type is the most detrimental to the chromatographic process whereas the former in certain cases may be beneficial, especially for weakly interacting systems.

The observed reinforced bioaffinity is most likely due to hydrophobic interactions between "oily" parts of the spacer and accessible hydrophobic regions at or near the ligand binding site of the enzyme. In agreement with this, it was found that binding of enzyme to the immobilized ligand was significantly reduced when hydrophilic extension arms were used (24) instead of the normally used aliphatic spacers. Low concentrations of an organic solvent, such as ethylene glycol, in the irrigant has also been reported to greatly improve the recovery of lactate dehydrogenase (LDH) by suppressing hydrophobic interactions in affinity chromatography of the enzyme on 5'-AMP-Sepharose (25).

Nonspecific hydrophobic binding in affinity chromatography may not only be due to interactions with aliphatic spacers but also to hydrophobic ligands or lipophilic groups on the ligand. "Hydrophobic chromatography" (26), which has become a recognized technique for purification of proteins, has taken advantage of such interactions although the matrix in this case has been derivatized with hydrocarbon chains, which also have been linked to negatively charged groups at the free ends of the hydrocarbon chains to minimize the potential risk of protein denaturation in the use of such columns (27).

Ionic nonspecific interactions effected by charged groups present on the spacer, the matrix or even the ligand itself are readily diminished (without affecting bioaffinity-binding) by using buffers of high ionic strength. Salt concentrations up to 0.5 M have been shown not to significantly influence K_D or K_i values for LDH (28).

4. Coupling of ligand to matrix

At present there are several methods available for attaching a ligand or a preassembled ligand to a matrix. The group that can be activated on the matrix can either be a hydroxyl, amino, carboxyl, carbonyl or a sulfhydryl function (10). CNBr-activation of the hydroxyl groups of beaded agarose (9) will only be considered here. The activation of the matrix sugar hydroxyls with CNBr is carried out at pH 11 for a short time; this procedure yields preferentially a reactive imidocarbonate (29) which efficiently couples with unprotonated primary amines. The predominating coupling product is a N-substituted isourea (30). The isourea linkage confers, however, a positive charge on the matrix at physiological pH (complications due to the ionic character of the gel have already been discussed above). Uncharged affinity adsorbents have also been prepared, for instance, by coupling the ligand through a dihydrazide to CNBr-activated agarose (31) instead of through a primary amine or to gels containing reactive epoxide groups (32).

The isourea linkage is sufficiently stable in most cases, however, in the presence of nucleophiles, detachment of bound ligands has been observed (33) and a slow release of ligands has also been detected at pH 5 and above (34). By coupling the ligand via a polyvalent spacer, such as polylysine, such leakage has been reported to be prevented (31). The CNBr-coupling procedure is also assumed to generate a uniformly substituted gel, however, activation and coupling conditions can influence the distribution of immobilized ligands throughout the matrix, especially when the ligand is a protein (35).

5. Binding capacity of affinity adsorbents

Besides the expected influence on the binding capacity of affinity adsorbents of experimental parameters such as pH, ionic strength and temperature (also used to effect elution) binding capacity has been shown to be a function of several other parameters. Thus, under column conditions, the capacity of 5'-AMP-Sepharose for LDH and glycerokinase has been shown to be related to the total amount of immobilized ligand (at constant ligand concentration) and also to the incubation time of the enzyme with the adsorbent (36). Under column conditions, the concentration of the applied enzyme had no effect on the binding capacity. However, under batchwise conditions binding capacity was related to the amount of the applied enzyme (36).

Binding capacity is also related to the length of the spacer (cf. p.13) and to the ligand concentration; a low substituted gel is less efficient than a highly substituted one. It is worth mentioning that commercially available 5'-AMP-Sepharose (Pharmacia, Sweden) which contains about 2 µmoles of ligand per ml settled gel can bind 2-3 mg of pure HLADH per ml of swollen gel in 0.1 M sodium phosphate buffer, pH 7.5, at 4°C (unpublished work).

An interesting and new approach to increase the binding capacity is to bind the complementary enzyme to the affinity adsorbent in the form of a strong dead-end ternary complex. This method to "lock" enzymes onto affinity adsorbents has been demonstrated in chromatography of LDH on NAD^+-Sepharose in the presence of oxalate (37). A similar approach was used to bind HLADH on low substituted NADH-Sepharose in the presence of the enzyme inhibitor isobutyramide (IV,VI).

Generally, only a small fraction (about 1%) of the immobilized ligands are effective in binding or retardation of enzymes (38). This may in part be due to unavailable ligand molecules which are coupled to portions of the matrix that are inaccessible to the enzyme and to screening by the bioaffinity-bound enzyme of neighbouring "free" immobilized ligands, thus preventing their interaction with

other enzyme molecules. The choice of matrix and also the selection of coupling density may be important considerations in the design of affinity columns of high relative binding capacity.

6. The elution step

Desorption from affinity columns can often be effected by changes in pH, ionic strength or temperature or, in the case of strongly interacting affinity systems, by high concentration of protein denaturants, such as urea or guanidinium hydrochloride. Desorption effected by electrophoresis is also possible (39). Such desorption methods are examples of nonspecific elutions. However, the bioaffinity-bound enzyme can also be eluted specifically with a ligand or an inhibitor or a combination of both which compete with the bound ligand for the enzyme. This latter type of elution or bioelution is commonly used in general ligand affinity chromatography, which allows a whole group of enzymes to interact with the immobilized ligand and, therefore, specificity in the elution step is often a requirement to obtain good resolution on the affinity column. The decreased selectivity of the adsorption step in general ligand or group-specific chromatography is thus compensated for by bioelution of the bioaffinity-bound enzyme.

An example of bioelution effected by binary complex formation is the separation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and LDH on NAD^+ - Sepharose (40). In this case, the two enzymes were separated by using short pulses of 0.15 mM NAD^+ and NADH, respectively. Resolution of a mixture of enzymes is normally not possible by using only a single pulse of the eluent and a more effective separation can be obtained by gradient elution. Thus, a mixture of the same dehydrogenases as above was efficiently resolved on 5'-AMP-Sepharose by using a linear gradient of NADH (41). Additional resolving power is gained if bioelution is effected by ternary complex formation. Thus, for instance, a pure preparation of LDH was obtained from a crude extract of ox heart in one step by specific elution of the enzyme from 5'-AMP-Sepharose with a short pulse of the dead-end complex-forming substrates NAD^+ and pyruvate (41). Other examples of enzymes purified to homogeneity by using ternary complex formation are alcohol dehydrogenase (I,II), citrate synthase (42) and phosphofructokinase (43). Ternary complex formation can be utilized not only as a means for specific elution but also to adsorb the enzyme strongly on the affinity column (cf. "locking-on", p.15). In this case, elution is effected by omission in the irrigant of one component involved in the complex. This method of elution, i.e. negative elution, has been applied in affinity chromatography of for example

alcohol dehydrogenase (IV,VI,44) and LDH (45).

From the above discussion it is evident that the ideal eluent in general ligand affinity chromatography should have the following characteristics: (a) it should be enzyme specific; (b) it should interact strongly with the enzyme to be eluted and (c) its rate of complex formation with the enzyme should be rapid. Examples of such eluents are reduced NAD^+-adducts (an adduct is a covalent complex between the coenzyme and the other enzyme substrate); these preformed adducts are specific inhibitors of specific dehydrogenases (46). Thus a mixture of LDH and malate dehydrogenase (MDH) was efficiently separated on 5'-AMP-Sepharose by specific elution with their respective reduced NAD^+ - adducts (47). An ideal eluent would be a transition-state analogue. Such analogues are very strong enzyme inhibitors and are also very specific (48).

IV AFFINITY CHROMATOGRAPHY OF HLADH ON IMMOBILIZED ADENINE NUCLEOTIDES

1. Preparation and characterization of affinity gels

Kinetic studies of a number of NAD^+-dependent dehydrogenases with N-hydroxyethyl derivatives of NAD^+ in free solution have indicated that modification of the N^6-amino group of NAD^+ results in no significant change in the binding of the coenzyme to these dehydrogenases (49). In agreement with this, X-ray crystallographic studies of NAD^+-dependent dehydrogenases (50) have shown that the coenzyme is generally bound to the enzyme in such a manner that the N^6-position of the adenine nucleus is pointing out from the protein surface into the solution and the C8-carbon atom is also oriented towards the surface but is not protruding out of the NAD^+ binding pocket as the N^6-amino group.

The affinity ligands used in the present work, 5'-AMP (I-III) and NADH (IV,VI), have each been condensed with spacers substituted in the N^6-position prior to their coupling to CNBr-activated Sepharose. As shown in Fig.2, the 5'-AMP-analogue, N^6-(6-aminohexyl) - 5'-AMP (having the spacer attached to the N^6-amino group in a stable alkyllinkage), was prepared from 6-chloropurine riboside in two steps involving phosphorylation and displacement of the halogen atom by 1.6-diaminohexane (51). The NADH-analogue, N^6-[N-(6-aminohexyl)carbamoylmethyl]-NADH was obtained from the corresponding oxidized coenzyme-analogue after enzymic reduction by yeast alcohol dehydrogenase. The N^6-substituted NAD^+-analogue had previously been prepared according to the following synthetic procedure (Fig.3): alkylation with iodoacetic acid at the N^1-position of the purine ring followed by Dimroth rearrangement in alkaline solution resulting in a carboxymethyl group

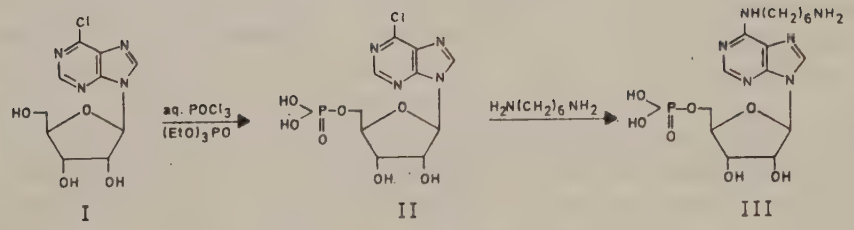

Fig.2. The synthesis of N^6-(6-aminohexyl)-5'-AMP (III). I = 6-chloropurine riboside; II = 6-chloropurine riboside phosphate.

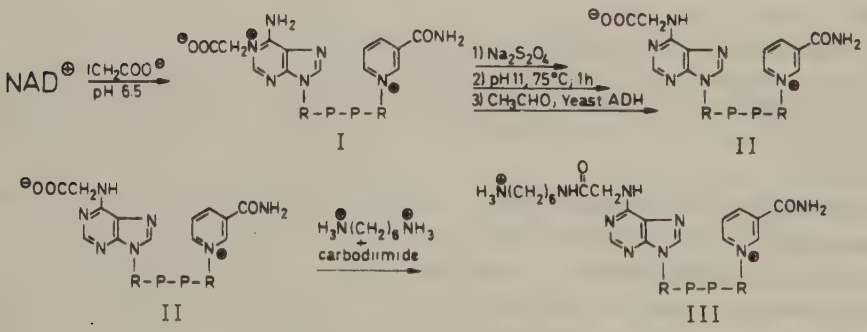

Fig.3. The synthesis of N^6-[N-(6-aminohexyl)carbamoylmethyl]-NAD$^+$ (III). I = 1-carboxymethyl-NAD$^+$; II = N^6-carboxymethyl-NAD$^+$.

at position N^6; this spacer was subsequently extended by condensation with 1,6-diaminohexane using a water soluble carbodiimide (52).

The nucleotide-analogues were coupled to Sepharose according to the CNBr-method (9). In the present study the amount of ligand-analogue immobilized was estimated from spectrophotometric analysis of the gel (I,IV,VI). The immobilized ligand concentration was in the case of 5'-AMP-Sepharose calculated from the absorbance at 266 nm (I) and in the case of NADH-Sepharose from the absorbance at 340 nm (IV,VI). In addition, it was found that the A_{340nm}/A_{266nm} ratio of the coenzyme-analogue (about 0.3) was not significantly changed after coupling the analogue to the matrix (unpublished work). The nucleotide

content was also determined after phosphate analysis of the freeze-dried affinity gel (II).

2. Purification and separation of HLADH on affinity gels

2.1. Purification from crude extracts

When HLADH has been purified from crude extracts with conventional methods, usually some of the following steps have been used: heat treatment of the aqueous liver extract, fractionation with ammonium sulphate ethanol-chloroform treatment, ion exchange chromatography and crystallization with ethanol. Although a pure enzyme in sufficient yield was obtained in these often time-consuming purifications, there has been need for an efficient purification of the enzyme in high yield preferentially in a single step procedure. To achieve this, a method was developed to purify HLADH from a crude liver extract on a column of 5'-AMP-Sepharose (I).

Pyrazole is a specific and very strong inhibitor of HLADH (53) and it is known to form a strong abortive ternary complex with the enzyme in the presence of NAD^+ ($K_{EPyr., NAD^+}$ = 0.1 µM at pH 7.0 (54)). This property of HLADH to form a complex with NAD^+ and pyrazole was exploited to elute the bioaffinity-adsorbed enzyme specifically from 5'-AMP-Sepharose (I). However, prior to elution of the bioaffinity-adsorbed enzyme it was necessary to wash the column with NaCl to remove weakly adsorbed proteins and also with a low concentration of NAD^+ to remove other nucleotide-dependent proteins which could otherwise have coeluted with HLADH in the subsequent specific elution with NAD^+ and pyrazole (Fig.4). Such a pre-wash with NAD^+ prior to specific elution with NAD^+ and pyruvate has also been reported to be efficient in the purification of LDH from ox heart crude extract on 5'-AMP-Sepharose (41).

Since pyrazole is a strong competitive inhibitor with ethanol (with a K_i of 0.2 µM at pH 7.0 (53)) it must be removed after elution of the enzyme from HLADH fractions before enzyme assaying. This can be achieved by dialysis (I) or gel filtration; a more efficient method is to apply those fractions containing HLADH on a column of hydroxyapatite to which the enzyme is adsorbed but not NAD^+ and pyrazole (55).

In this one-step purification of HLADH on 5'-AMP-Sepharose electrophoretically homogeneous enzyme was obtained (I), although the enzyme was not separated into its isozymes. Furthermore, it is possible to prepare pure HLADH by this method in one day. Elution effected by ternary complex formation involving

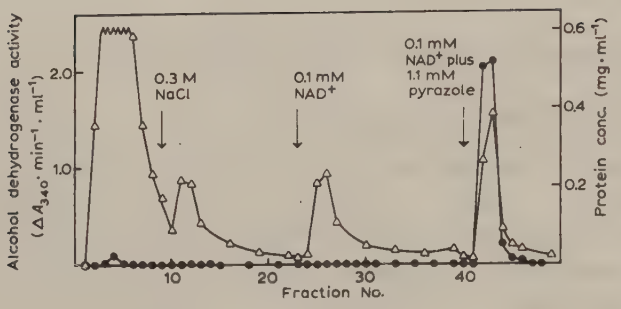

Fig.4. Purification of HLADH from a horse liver crude extract on a column of 5'-AMP-Sepharose by elution with NAD^+ and pyrazole. (●—●), HLADH activity; (△—△), protein concentration.

enzyme, NAD^+ and pyrazole has also been successfully employed in the purification of chicken liver (56) and rat liver alcohol dehydrogenase (55) on 5'-AMP-Sepharose.

In all the above pyrazole was used as specific eluent for alcohol dehydrogenase. It can also be immobilized to Sepharose and used as a specific adsorbent for the enzyme. Thus it was found that alcohol dehydrogenase is strongly adsorbed on matrix-bound 4-[3-(N-6-aminocaproyl)aminopropyl]-pyrazole in the presence of NAD^+ (44). In this specific ligand approach the enzyme was eluted by adding ethanol, propanol or butanol to the NAD^+-containing buffer to form a second strong ternary complex competing with the first one. This "double ternary complex affinity chromatography" has been employed with just one other purification step on DEAE-cellulose to isolate and purify large quantities of alcohol dehydrogenase from human, horse, rabbit and rat livers (44).

A number of affinity systems are listed in Table 1 that have in recent years been utilized in the affinity chromatography of HLADH. Noteworthy is the use of blue dextran-Sepharose (63). In this case the blue dye (commercially known as Cibracron Blue F3GA) in blue dextran is assumed to bind the enzyme by interacting specifically with a unique part of the enzyme structure called the dinucleotide fold (the AMP binding domain) which is found among several nucleotide binding proteins such as dehydrogenases, kinases and other nucleotide-dependent proteins (64).

TABLE 1.

Affinity systems used in model studies and for purification and isolation of HLADH

Ligand	Point of attachment of immobilized ligand	Reference(s)
NAD(H)	N^6	IV,VI,24,52,57
NAD^+	C8	24,28
NAD^+	ribose	28,58
5'-AMP	N^6	I,II,47,58,59,60
"	C8	61
"	ribose	58
NMNH[a)]	phosphate	62
Pyrazole	C4	44
Blue dextran		63

a) Nicotinamide mononucleotide (reduced form).

2.2. Separation of isozymes

Isozymes are defined as multiple subunit enzymes which have similar or identical enzymic activities and the multiplicity must be due to genetically determined differences in the primary structure (65). Dimeric HLADH has three isozymic forms, usually termed EE, ES and SS (66); the primary structures of the two subunits E and S are very similar and a difference of only six amino acid residues has been found by peptide mapping (67). Additional multiplicities of each isozyme are also known (68) as well as a polymorphic form of the enzyme (69). The isozymes differ in substrate specificity. All three are active with ethanol as substrate, whereas only isozymes with the S-subunit are active with certain 3β-hydroxy steroids (70). In addition, 3α-hydroxy steroid derivatives are potent inhibitors of the enzyme. Litocholic acid (3α-hydroxy-5β-cholanoic acid), for instance, is strongly competitive with 3β-hydroxy steroid substrates of both ES (71) and SS (69). The main isozymes have been purified with conventional methods; for example, EE as described in reference (72), ES as in reference (71) and SS as in reference (73).

Model studies. Paper I describes a method to separate the main isozymes of HLADH by affinity chromatography. A mixture of the EE and SS forms were applied to a column of 5'-AMP-Sepharose and the isozymes were bound. It has been concluded that true bioaffinity-binding had taken place since no isozyme showed affinity for the control gel, n-hexylamine-substituted Sepharose 4B (I).

Afterwards, on application of a linear gradient of NAD^+ in the presence of cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid) the SS form was eluted first and was well separated from the EE form (Fig.5). It has been suggested

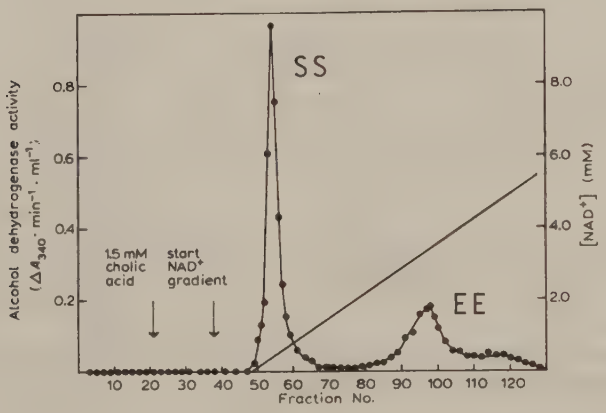

Fig.5. Separation of a mixture of the EE and SS isozymes of HLADH on a column of 5'-AMP-Sepharose by elution with NAD^+ and cholic acid. (●—●), HLADH activity.

that elution of HLADH was effected by ternary complex formation of enzyme with NAD^+ and cholic acid, and with the latter, cholic acid, preferentially binding to the isozyme with the greatest steroid activity. This is supported by the following reported observations: (a) in an identical chromatographic procedure as described above on 5'-AMP-Sepharose but with the omission of cholic acid poor resolution of the isozymes was obtained; (b) the order of biospecific elution of EE and SS is in accordance with their sensitivity to their inhibition by 3α-hydroxy steroids; and (c) cholic acid alone did not elute any isozyme. Ternary complex formation has also been used to separate LDH from MDH on a column of ADP-Sepharose (74) in a procedure analogous with the one used in paper I by including either pyruvate or malate in the NAD^+-gradient. In the former case LDH was eluted first and in the latter MDH.

The reported dissociation constant between NADH and enzyme is 0.28 μM and 0.09 μM for EE and SS, respectively, at pH 8.0 (75). Elution with various gradients of NADH at pH 7.5 did not separate EE and SS on the 5'-AMP column (I). This is noteworthy especially in view of the fact that a difference in K_D (for NADH) by a factor of 4 between ox LDH isozymes was sufficient for their effective

separation on 5'-AMP-Sepharose (76). In this case, elution was effected by a concave gradient of NADH (at pH 7.5). However, in this connection it has to be noted that it is not only the interaction between enzyme and coenzyme that is important for the separation on the column but also the interaction between enzyme and immobilized ligand.

Preparative purification of the homogeneous steroid-active isozyme. Based on the successful separation of EE and SS in the model studies on 5'-AMP-Sepharose (I) a method was developed for the preparative purification of SS directly from a crude extract using affinity chromatography (II). The method involves the following main steps: batchwise fractionation on CM-cellulose of horse liver crude extract, and chromatography (twice repeated) on 5'-AMP-Sepharose. In both affinity steps SS was eluted specifically by a pulse of NAD^+ and cholic acid. Prepurification on CM-cellulose was introduced not only to lower the amount of protein but also to reduce the concentration in the crude extract of EE and ES (both normally present in excess of SS in the original extract (II, 73)) which compete with SS for the immobilized ligand. From 260 grams of horse liver 30 mg of electrophoretically pure SS could be isolated. Recently, specific elution of SS from 5'-AMP-Sepharose with NAD^+ and cholic acid in the buffer has also been employed as a purification step in the isolation of the homogeneous steroid-active isozyme from S-type horse livers (60).

Since affinity columns of the type used in the present study will adsorb a large number of dehydrogenases and kinases and other nucleotide-dependent proteins it has been argued (44) that such columns will preferentially bind enzymes present in crude extracts with the most favourable binding constants, resulting in a poor binding capacity of the column for weakly interacting enzymes or enzymes present at low concentrations in the extract. This may be true, but, as demonstrated in paper II, such problems connected with crude extract purifications on general ligand adsorbents may often be readily solved by prepurification of the extract with conventional methods so that the capacity of the affinity adsorbent can be better utilized. Rechromatography of partially purified enzymes on the affinity column can also be used.

Isozymes of several dehydrogenases other than LDH and HLADH have also been separated by affinity chromatography. For example, MDH isozymes from watermelon cotyledons were separated and purified on 5'-AMP-Sepharose (77) and lipoamide dehydrogenase isozymes from pig heart were partially resolved on

NAD^+-Sepharose (78). Furthermore, large-scale purification of M_4 LDH from dog fish on 5'-AMP-Sepharose has been carried out (47) as well as preparative purification of H_4 from pig heart on a column of immobilized oxamate (79). Using the latter adsorbent LDH-X was separated from other isozymes of mouse testis (80). This system has also been used to differentiate between M_4 and H_4 isozymes of LDH of human placenta (81). On a column of 8-(6-aminohexyl)-amino-2'-AMP substituted Sepharose 4B, it was possible to separate hybrid hexamers of $NADP^+$-specific glutamate dehydrogenase from Neurospora (82).

2.3. Application of magnetic affinity material for purification and separation of HLADH

Magnetic polymers have recently been used as support material for biological molecules. The inherent advantage in the use of such polymers is, in particular, the quick and easy retrieval of the polymers by applying an external magnetic field. Derivatized magnetic polymers have been applied in the fields of immunochemistry (83) and immobilized enzymes (84) and also as affinity adsorbents for purification of enzymes (III,15).

Magnetic affinity adsorbents have been prepared by different methods. The ligand can be attached through a spacer either to magnetic particles which previously have been coated with a thin layer of polyacrylamide or directly to iron oxide particles. A serious limitation in the use of such magnetic adsorbents is, however, that they have a low binding capacity for the complementary enzyme and that nonspecific binding occurs to the support material per se.

In paper III there is an alternative method reported for preparing magnetic affinity adsorbents. This method is based on "post-magnetization" of polymers already substituted with ligands. The affinity material is "magnetized" by continuously pumping a magnetic fluid through a column packed with the adsorbent. (The fluids used, that is ferrofluids, can be characterized as fluids consisting of ultramicroscopic ferrite particles, about 100 Å, which are stabilized by a coating about 25 Å thick, preventing their sticking together in a magnetic field.) On this treatment affinity gels, which retained their biospecificity, were obtained and these had excellent magnetic properties and high binding capacity for their complementary enzymes. The mechanism of magnetization has not yet been established, although adsorption of ferrite particles to the gel beads or precipitation of the particles in the interior of the beads are likely explanations. Poor enzyme recoveries were obtained after chromatography on freshly prepared magnetic adsorbents, indicating a possible irreversible

binding of enzyme to the gel-ferrite assembly. However, recoveries of the enzyme were greatly improved by treatment of the magnetic adsorbents with bovine serum albumin (BSA) prior to their use. Porous glass affinity adsorbents have also been coated with BSA after ligand attachment to block non-specific adsorption sites (85).

Batchwise affinity chromatography of HLADH on magnetized 5'-AMP-Sepharose demonstrated that both isozyme fractionation using NAD^+ and cholic acid and purification from crude extracts using the NAD^+-pyrazole elution technique was possible (III). In another model experiment described in paper III it was shown that HLADH could be isolated within minutes after adding magnetized 5'-AMP-Sepharose to a quickly prepared liver extract; this demonstrates the potential use of the technique for quick retrieval of labile enzymes that are particularly susceptible to degradation by proteolytic enzymes.

3. Some binding studies of HLADH using affinity gels

Although affinity chromatography today has proven to be a highly useful technique for the purification of enzymes much has yet to be learned about the nature of the interaction between the immobilized ligand and the complementary protein. For instance, is the binding of the enzyme to the ligand on solid-phase identical to the binding of the ligand in free solution? In most cases this is probably the case, but nonspecific factors such as ionic and hydrophobic interactions may play a major role in the binding process on the matrix (21,22) (cf. p.13).

Criteria that are often used to show that biospecific interactions are taking place on solid-phase are: (a) the low concentrations of added ligands (μM-mM range) required to elute the enzyme from the affinity adsorbent; (b) similar dissociation constants found for the free and bound ligand (86); and (c) retained biological activities of immobilized coenzyme-analogues (52). Other observations have also been made which strongly indicate that the enzyme-ligand interaction on the matrix must be very similar to the interaction that takes place in homogeneous solution. For example, it was found that $NADP^+$-dependent dehydrogenases are preferably bound to 2',5'-ADP-Sepharose (2'5'-ADP is part of the $NADP^+$ molecule) rather than to 5'-AMP-Sepharose (59). The opposite situation was found for NAD^+-dependent dehydrogenases which showed the greatest affinity for the 5'-AMP-Sepharose (5'-AMP is part of the NAD^+ molecule) (59). Similarly, the CoA-dependent enzyme succinate thiokinase was found to interact much stronger with 3',5'-ADP-Sepharose (3',5'-ADP is part of the coenzyme A

molecule) than with 2',5'-ADP-Sepharose or 5'-AMP-Sepharose (87).

3.1. Enzyme-coenzyme interactions on solid-phase studied by spectrofluorometry.

The fluorescence of NADH is known to increase significantly when the coenzyme is bound to HLADH either in the form of a binary complex with the enzyme alone or in the form of a ternary complex with the enzyme and for instance the enzyme inhibitor isobutyramide (88,89). In paper IV a method, based on spectrofluorometry, is reported and involves the study of the interaction between HLADH and the immobilized NADH-analogue, N^6-[N-(6-aminohexyl)carbamoylmethyl]-NADH. For the fluorescence measurements a special flow-cell developed for solid-phase fluorometry was used (90). In order to restrict enzyme-coenzyme interactions on the matrix (Sepharose 2B) to a single subunit low substituted NADH-Sepharose was prepared (about 20 nmoles NADH-analogue per ml of settled gel; this ligand concentration is roughly a factor of 100 less than that utilized in ordinary affinity chromatography). It should be mentioned that the spectrofluorometric method described in paper IV is similar to that utilized in the study of conformational changes taking place in immobilized enzymes (90,91). The fluorescence spectra observed in this study indicated that the immobilized NADH-analogue could form both a binary complex with HLADH alone and a ternary complex with HLADH plus isobutyramide (Fig. 6).

Based on the fluorometric studies reported in paper IV alone, nothing can be said as to what extent biospecificity was involved in the interaction between enzyme and the immobilized coenzyme-analogue since no quantum yields were estimated. It can, nevertheless, be suggested that the very good agreement between the spectra of the immobilized systems and those of the corresponding soluble ones must reflect that the binding on solid-phase has to be very similar to the binding in free solution.

It would be interesting to use the fluorometric method described in paper IV to study the interaction on solid-phase between HLADH and immobilized NADH when the coenzyme is coupled to the matrix via a hydrophilic extension arm. Such studies would clarify why dehydrogenases are bound significantly weaker to ligands when attached through hydrophilic extension arms. If the weak binding is due to inaccessibility of the ligand caused by hydrogen bonding between the hydrophilic matrix and the hydrophilic extension arm as suggested in reference (92) this would probably result in no fluorescence enhancement, for instance, when HLADH is allowed to interact with the affinity gel in the presence of isobutyramide. On the other hand, if the weaker binding is due to decreased

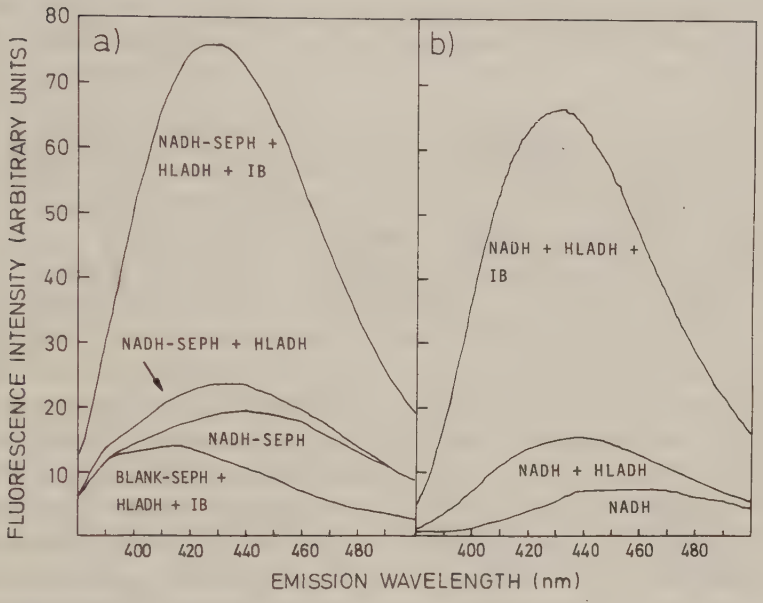

Fig.6. (a) Fluorescence emission spectra obtained with NADH-Sepharose. (b) Fluorescence emission spectra obtained with soluble NADH. The excitation wavelength was 330 nm. IB = isobutyramide.

nonspecific interaction between the bioaffinity-bound enzyme and the hydrophilic extension arm (24) the fluorescence of the interaction between HLADH and immobilized NADH when attached either via an aliphatic or a hydrophilic extension arm should not differ significantly.

3.2. Affinity chromatography on low substituted NADH-Sepharose

Binding studies. Biospecific binding was also investigated by affinity chromatography of HLADH on the low-substituted NADH-Sepharose (IV). It was found that HLADH was strongly adsorbed on the NADH-gel in the presence of isobutyramide, but was not bound when the amide was omitted from the buffer (in the latter case the enzyme was only retarded on the NADH-gel). These results agree well with the affinity pattern that can be predicted from K_D values reported for NADH dissociation from the enzyme-NADH-isobutyramide ternary complex (5.5×10^{-9}M at pH 7.0, 23.5°C (89)) and the enzyme-NADH binary complex (0.25×10^{-6}M at pH 7.0, 8°C (93)). It was also found that dialkylated HLADH (obtained after modification of HLADH by iodoacetic acid) was not bound to the NADH-Sepharose even in the

presence of isobutyramide. This is in accord with the suggestion that dialkylated enzyme is not readily capable of forming ternary complexes with NADH that involve either aldehydes or enzyme inhibitors such as acetamide or isobutyramide (94).

Quantitative affinity chromatography. Affinity chromatography has been successfully used to evaluate dissociation constants for binding of enzyme to soluble or immobilized ligands. For this purpose, both zonal (23,37,86) and frontal (95) affinity chromatographic experiments have been carried out; a method based on gradient affinity chromatography has also been reported useful for such binding studies (96). From the results of frontal affinity chromatography on the low substituted NADH-Sepharose (the column, 1.3 x 3.8 cm, was packed with the same gel as described in paper IV) carried out in 0.1 M sodium phosphate buffer, pH 7.5, at a flow rate of 16 ml/hr and at $4^{o}C$, it was possible to estimate an intrinsic K_D of approximately 2μM - according to the method reported in reference (95)-for the binding of HLADH to the immobilized NADH-analogue (unpublished work). This value agrees with the kinetic K_D value (see above) for binding of NADH to HLADH in free solution and supports the suggestion made in paper (IV) that the nature of the binding on solid-phase must be roughly identical to that in free solution. It is interesting to note that compound affinity due to nonspecific interactions between the bioaffinity-bound enzyme and the extension arm does not seem to play a major role in this system which has been reported to be the case for HLADH when subjected to affinity chromatography on NAD^{+}-Sepharose (24).

It is suggested that chromatography on low substituted affinity gels should provide a suitable means to estimate binding constants for interactions between enzyme and immobilized ligand (provided that the enzyme is retarded on such columns) as indicated in this thesis. Such studies have also been successfully carried out to determine the dissociation constant for binding of colipase to Sepharose-bound lipase and vice-versa (97).

V ASPECTS OF SUBUNIT INTERACTIONS IN HLADH

Oligomeric proteins are proteins which consist of two or more polypeptide chains or subunits arranged in a specific manner to form a quaternary structure. The quaternary structure may have several different functions in different proteins. For example, a possible role of the oligomeric structure could be to confer additional stability on the subunit assembly compared with that of an individual subunit as indicated in studies of tetrameric aldolase from rabbit muscle (98).

An interesting suggestion about the role of the oligomeric structure is that subunit association may be necessary to keep the osmotic pressure in the living cell at an acceptable level (99). In addition, allosteric properties of many regulatory enzymes have been interpreted successfully in terms of cooperative interactions between subunits (100). Other functions have also been ascribed to enzyme subunit systems (101). Nevertheless, the relationship between quaternary structure and enzyme activity is difficult to explain for a large number of oligomeric enzymes which show classical Michaelis-Menten kinetics. For example, is the oligomeric structure essential for their activity, and if so, is there also in these enzymes cooperative interactions between subunits during catalysis. HLADH belongs to this latter category of oligomeric enzymes.

HLADH catalyzes a two-substrate reaction. The enzyme shows classical Michaelian kinetics and it also operates by a sequential mechanism of Theorell-Chance type in which the binding of the coenzyme (NAD(H)) preceeds the binding of the substrate (alcohol or aldehyde) (102) but oxidation of secondary alcohols is not consistent with an ordered mechanism (103). The binding of coenzyme, which results in a conformational change (104), shows no evidence for any type of cooperative ligand binding (105).

Stopped-flow kinetic studies have shown that the reduction catalyzed by HLADH of various aromatic aldehydic substrates - above pH 8 and under single-turnover conditions - follows a transient time-course that is biphasic (i.e. it consists of two-steps, a fast and a slow, that are equal in amplitude) (106,107). On the basis of this transient kinetic pattern, it was suggested that two subunits of HLADH are kinetically nonequivalent. The first and fast step of the transient time course may indicate a chemical transformation in one subunit and the slow step (in the time course) chemical transformation in the other subunit but coupled with product diffusion from the first reacted subunit ("half-site reactivity"). Based on this proposed negative cooperativity between the subunits a mechanism involving alternately functioning subunits has also been suggested for HLADH (108). (Such an enzyme mechanism has been called a flip-flop mechanism and is suggested to be consistent with Michaelis-Menten kinetics (109).) Furthermore, X-ray crystallographic studies have shown that the symmetry associated with the apoenzyme is lost in crystals when the coenzyme is bound to HLADH (110). This fact has been used as an additional evidence for half-site reactivity. It has also been suggested that nonequivalent substrate binding sites could be formed in HLADH on coenzyme binding (7). On the other hand, there are several other reports which, in contrast to the above mentioned,

state that the presteady-state kinetic behaviour of HLADH is in fact in accordance with kinetically identical subunits and is not evidence for half-site reactivity of the enzyme (111-113).

A number of other enzymes such as alkaline phosphatase from Escherichia coli and mitochondrial MDH, which are dimeric enzymes and show Michaelian kinetics, have been considered to show half-site reactivity (109,114), but again some other reports conflict with this explanation (115,116). In the case of GAPDH evidence for half-site reactivity is more clear cut although different explanations for the phenomenon have been proposed (117).

VI THE USE OF BIOCHEMICAL SOLID-PHASE TECHNIQUES IN THE STUDY OF SUBUNIT INTERACTIONS IN HLADH

The immobilization technique has proven of value as an aid in answering questions that concern relationships between quaternary structure and enzyme activity (118). The use of this technique in the study of structure-function relationships in dehydrogenases and to investigate allosteric regulation is reported in reference (119). The purpose of the investigation presented in paper V was to study whether immobilized single HLADH subunits are enzymically active or whether they can regenerate activity by interacting with soluble subunits. Paper VI describes a method to prepare monoalkylated HLADH, enzyme consisting of one active and one inactivated subunit. The object here was to investigate whether cooperative interactions between the subunits of HLADH are essential for catalysis.

1. General methodology in the preparation of immobilized subunits

The normal method of preparing immobilized subunits involves coupling the oligomeric protein in such a manner that only one monometer would be covalently linked to the matrix. Subsequent treatment of the obtained matrix-bound protein with a protein denaturant such as urea (V,98,120) or guanidinium hydrochloride (121-124) removes noncovalently bound subunits and yields immobilized subunits. Furthermore, on a return to nondissociating conditions, which brings about refolding of the covalently linked subunits, there is no risk of any spontaneous reassociation of the subunits if they are immobilized far enough apart from each other on the matrix.

The requirements for a matrix are the same as those desired for the bed materials in affinity chromatography and also a similar coupling technique can be used for subunit immobilization as that utilized for the preparation of affinity

adsorbents (cf.p.10 and 14). However, in order to increase the probability that only a single subunit of the oligomer is covalently attached to the matrix it is necessary to limit the number of activated groups on the support material and also to add a relatively high concentration of the protein to be immobilized to the activated matrix. In the case of Sepharose, a suitably "diluted" matrix is usually obtained by using a very low concentration of CNBr, usually less than 5 mg per ml of settled gel (V,98,120-124). The lowest possible concentration of CNBr that can be used is determined by the turnover number of the oligomer to be immobilized and the amount of protein that can be determined on the matrix with sufficient accuracy.

Another possible method of preparing matrix-bound subunits is coupling the monomeric form directly to the matrix under dissociating conditions. However, in this case there is a pronounced risk that coupling may occur through an amino acid residue that is important for the folding of the enzyme or is located at or near the subunit contact area (118).

The protein concentration remaining on the gel after dissociation is usually determined to establish whether immobilized single subunits had been produced (V,98,120-124). Specific interaction of matrix-bound subunits with added soluble subunits is another criterion often used to show the existence of immobilized subunits (V,98,120-124). If, for instance, the bound monomeric form is inactive, addition of soluble subunits might be expected to induce activity in the bound subunit and the regenerated bound activity should be close to that shown by the immobilized oligomer. However, the extent of reassociation may be limited by several factors. For example, bound subunits may become inactivated due to irreversible conformational changes caused by the drastic conditions required for dissociation of the oligomer. It is also possible that the somewhat heterogenous population of immobilized subunits may have an effect on reassociation both qualitatively and quantitatively.

2. Dissociation of immobilized HLADH in concentrated urea

HLADH was coupled to weakly CNBr-activated Sepharose 4B (V). After dissociation with 6 M urea the preparation had lost practically all of its bound activity (more than 90%). However, on this treatment roughly 24% of the protein content was removed, which has been interpreted that about every second protein molecule was linked via both subunits to the matrix. This would imply that even though a weakly activated gel was used there is still a risk of multiple-point attachment of at least a fraction of the coupled protein.

Immobilized subunits contaminated with bound oligomeric nondissociating protein have also been observed in other studies (122,123); by aid of statistics the extent of multiple-point attachment could be related to the number of activated groups on the matrix (121). Other oligomeric enzymes (98,120,124), likewise coupled to weakly CNBr-activated Sepharose, could indeed be completely dissociated into monomers on solid-phase. It is possible that the nature of the oligomer - i.e. the number of accessible reactive groups, usually lysines, on the protein surface as well as protein shape and size - may influence the mode of attachment and affect immobilization.

The remaining enzyme activity of the urea-treated immobilized enzyme preparations described in paper V has been suggested to come from the single dimers linked to the matrix via both subunits. On the basis of this and the fact that enzyme activity could be regained after addition of soluble subunits (see below for more detailed discussion) it has been concluded that matrix-bound single subunits of HLADH are inactive. This would be in agreement with the known three-dimensional structure of the enzyme which shows that both subunits contribute amino acid residues to each active-site pocket (7). Furthermore, it has been suggested that correct refolding of denatured monomers of oligomeric dehydrogenases seems to occur first upon their reassociation to oligomers (125). In agreement with this, it was found that Sepharose-bound LDH subunits are inactive (123).

Examples of Sepharose-bound enzymes shown to be active in their immobilized monomeric forms are rabbit muscle aldolase (98), transaldolase from Candida utilis (122) and creatine kinase from rabbit muscle (124). For the first two mentioned enzymes it was found that the urea concentration required for half-inactivation of the immobilized subunits was significantly lower than that required to inactivate the corresponding matrix-bound native enzyme. Thus, stability to urea treatment has been suggested as a tool for distinguishing between active bound single subunits and matrix-bound oligomers (118).

3. Reassociation on solid-phase to form hybrid HLADH

In a study of matrix-bound glycogen phosphorylase it was shown that added soluble subunits of phosphorylase previously inactivated by modification in the active site could induce activity in Sepharose-bound monomers (126). Thus regeneration of the activity of immobilized enzyme offers firstly strong experimental evidence for the existence of immobilized monomers and secondly that matrix-bound subunits are not rendered inactive due to their coupling to

the matrix per se. A similar approach is described in paper V, but instead of adding chemically modified subunits the matrix-bound monomers were allowed to interact with soluble subunits of an isozyme having a different substrate specificity. The principal procedure used to prepare hybrids from subunits of the immobilized SS isozyme and soluble subunits of the EE isozyme is outlined in Fig.7. It was found that the urea-treated Sepharose-bound SS isozyme on

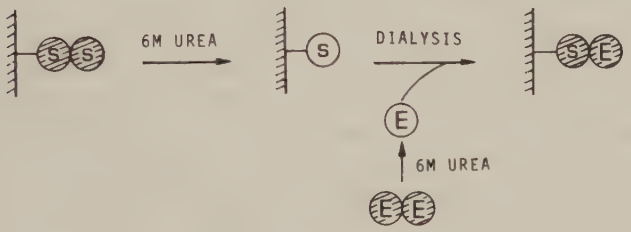

Fig.7. Reaction scheme for dissociation of the immobilized steroid-active SS isozyme of HLADH with urea, followed by regeneration of steroid activity on addition of soluble E-subunits. Shaded circles denote activity.

addition of soluble E-subunits regained about 35% of the activity of the untreated immobilized SS form (dimeric EE is not active with the steroid substrate 5β-dihydrotestosterone used (V)). In this study, great care was taken to reduce contaminating steroid activity in the EE preparations by chromatography of these preparations on 5'-AMP-Sepharose as reported in papers I and II. It was also found that induction of activity requires interaction between homologous subunits since other proteins such as LDH or BSA were not effective in regenerating activity of immobilized enzyme (preliminary studies have indicated that native yeast alcohol dehydrogenase was also very poor in this respect), in addition no significant enzymic activity could be observed after addition of soluble subunits to reference Sepharose (i.e. CNBr-activated Sepharose to which no protein had been coupled).

Noteworthy is the observation, described in paper V, that steroid-active enzyme was obtained when soluble native EE was added to immobilized S-subunits even in the absence of any denaturant. Furthermore, carboxymethylated dimeric EE (virtually inactive EE form (94)) was found to be effective as well. Two explanations for this observation have been proposed: (a) association of bound

subunits with soluble subunits generated *in situ* in the absence of any denaturant; and (b) association of an immobilized subunit with one or more soluble dimers forming an aggregate which after rearrangement could dissociate to form immobilized dimers. Although the existence of single HLADH subunits under non-dissociating conditions has not been observed (127), available data cannot be used to favour either of the two suggested mechanisms.

4. Preparation of monoalkylated HLADH on solid-phase

A possible way to test whether the catalytic mechanism of HLADH involves alternately functioning subunits is to prepare and study an enzyme molecule in which only one subunit can function catalytically. Therefore, a method was developed to prepare hybrid HLADH inactivated in a single subunit (VI). The method involves carboxymethylation of the enzyme while it is strongly bioaffinity-bound in the presence of isobutyramide to the immobilised NADH-analogue, N^6-[N-(6-aminohexyl)carbamoylmethyl]-NADH (Fig.8). Iodoacetic acid is a suitable reagent for such a purpose since it is known to inhibit HLADH by selectively reacting

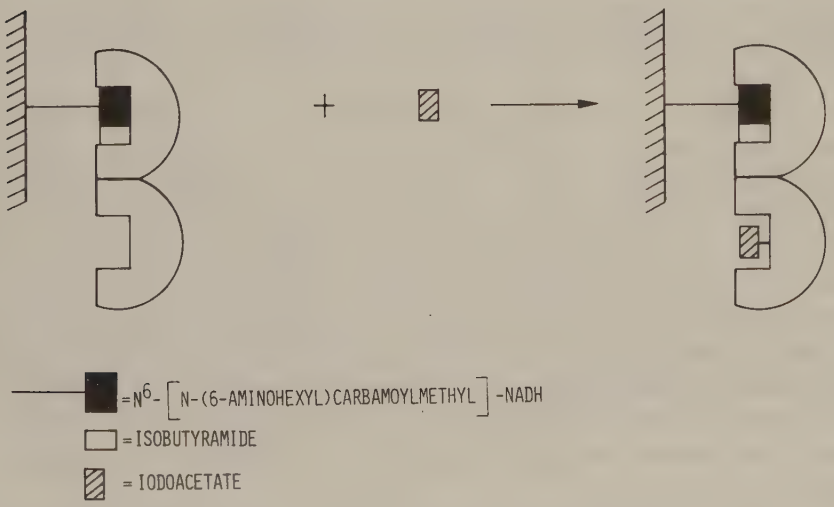

Fig.8. Schematic illustration of the carboxymethylation of HLADH while the enzyme is bioaffinity-bound to NADH-Sepharose in the presence of isobutyramide.

with Cys-46 in the two active site regions (94) and in doing so no significant

conformational change appears to occur in the protein structure (128). Studies on hybrids formed from subunits of a native enzyme and subunits of an inactive oligomeric variant (produced either by a point mutation or by selective modification with a site-specific reagent) have provided insight into the question of whether there are cooperative subunit interactions in other multisubunit enzymes (115,129).

The same NADH-Sepharose was used to prepare monoalkylated HLADH (VI) as that employed to study enzyme-coenzyme interactions on solid-phase (IV). After alkylation on solid-phase the enzyme was eluted from the affinity adsorbent and studied in free solution. Results of the incorporation of radioactive iodoacetic acid into bioaffinity-bound HLADH strongly indicate that a single subunit of HLADH was alkylated. Also, the specific activity with ethanol as substrate (half of that of the native enzyme) and the remaining number of NADH-binding sites per enzyme molecule (about 50%) - determined fluorometrically by titrating with NADH in the presence of isobutyramide (89) - suggest that the reaction with iodoacetic acid had to a great extent taken place at the active center of the "free" (not bioaffinity-bound) subunit. Furthermore, amino acid analysis of the monoalkylated enzyme also showed that S-carboxymethylcysteine had been formed.

Since the enzymic reduction of benzaldehyde has been interpreted as showing half-site reactivity in HLADH (106-108), steady-state kinetic parameters were determined for this reaction for both native and monoalkylated enzyme. The data obtained at pH 8.6 clearly demonstrate that neither the K_m nor the enzyme turnover number (monoalkylated HLADH showed half the specific activity of native enzyme) was significantly changed on alkylation (Table 2). These results may

TABLE 2.

Steady-state kinetic parameters determined for the reduction of benzaldehyde by NADH with native and monoalkylated HLADH[a)]

Preparation	$V/[E]$[b)] (s^{-1})	K_m (μM)
Native HLADH	9.9 ± 0.2	33 ± 1
Monoalkylated HLADH	10.6 ± 0.4	32 ± 2

a) The measurements were carried out at 26°C in 0.1 M glycine-NaOH, pH 8.6, at saturating concentration of NADH (90 μM).

b) The turnover number was calculated on the basis of the enzyme normality, estimated after titration of the enzyme with NADH in the presence of isobutyramide (89).

indicate that the two subunits of native HLADH are kinetically equivalent (for a more detailed discussion on this see General Discussion).

An interesting observation is described in paper VI. It was found that HLADH gradually lost both activity and its ability to form a ternary complex with NADH and isobutyramide when the bioaffinity-bound HLADH was incubated in buffer containing isobutyramide only. After the length of time usually used for carboxymethylation the specific activity and the NADH-binding capacity in the presence of isobutyramide of this preparation was found to be roughly 50% of that of untreated enzyme. It has been suggested that this observation might reflect inactivation of the "free" (not bioaffinity-bound) subunit; it is known that HLADH in the form of a ternary complex with NADH and isobutyramide is significantly more stable than free enzyme, for example, against heat inactivation or inactivation due to extremes in pH (130). It should be mentioned that enzyme was inactivated much slower in free solution (that is HLADH dissolved in buffer only or buffer containing isobutyramide) under the same conditions as on solid-phase (VI). Further studies are required to understand the nature of this inactivation on solid-phase.

VII GENERAL DISCUSSION

The present study shows that general ligand affinity chromatography has potentially wide application in the study of HLADH. Thus this technique should not only be useful as a means for efficient and rapid isolation and purification of HLADH but also in studying interactions between the enzyme and immobilized ligands.

Other possible applications of affinity chromatography in the study of HLADH could be screening of new inhibitors of the enzyme and in discovering dead-end ternary complexes in which HLADH is involved as demonstrated in the separation of the EE and SS isozymes on 5'-AMP-Sepharose (I). Furthermore, it has been shown that it is theoretically possible to estimate rate constants with affinity chromatography (131). The use of affinity chromatography for such purpose might be a good complement to the stopped-flow technique which is normally used to determine the rate of ligand binding to and dissociation from HLADH.

As indicated above, affinity chromatography offers several possibilities for studying HLADH. However, the enzyme can also be studied when covalently bound to a solid support. A number of such studies have been carried out. For

example, the effect of immobilization on the active site integrity of HLADH has been investigated (132) as well as the thermal stability of matrix-bound HLADH using calorimetry (133). HLADH has also been coimmobilized with a NADH-analogue (the same coenzyme-analogue as was used in papers IV and VI) to Sepharose in such a manner that the coenzyme-analogue was permanently positioned in the active center of the matrix-bound enzyme (134). It was demonstrated that this preparation was active in an assay system without requirement for any added soluble coenzyme.

In the present study immobilized single subunits of HLADH were prepared in order to answer questions such as the relationship between the quaternary structure and the enzyme action of HLADH (V). The successful formation of hybrids between immobilized inactive S-subunits and soluble E-subunits not only shows that immobilized HLADH subunits are inactive in themselves but also that they are capable of specifically "picking up" soluble subunits. It is, therefore, concluded that dimer formation is a prerequisite for HLADH activity; a conformational change appears to occur in the inactive subunits on reassociation of the enzyme so that activity is regained. Furthermore, the observed regain in enzyme activity, which was obtained when bound subunits interacted with added soluble dimeric enzyme (V), suggests that true subunit exchange was taking place on solid-phase.

In a recent study the effect of Zn^{2+} on the refolding and reactivation of HLADH in free solution was investigated (135). The results of this study suggest that zinc is required to initiate association of dissociated subunits. However, an excess of zinc leads to a significant decrease in the yield and the rate of the reactivation process. This was also found to be the case when immobilized single subunits of HLADH were allowed to interact with soluble subunits in the presence of added Zn^{2+} (V). Thus, zinc ions may be essential not only for activity and stability of HLADH but also for reconstitution of the enzyme from its dissociated state.

It is proposed that the immobilization technique described in paper V should be applicable in constructing hybrids made up of matrix-bound HLADH subunits and soluble subunits of other alcohol dehydrogenases. Such studies should provide information on whether and to what extent intersubunit contact areas have been conserved in the evolution of different molecular species of alcohol dehydrogenase. Analogous hybridization experiments carried out on solid-phase have provided important information about subunit interactions in other oligomeric enzymes. For instance, stable hybrids have been constructed between inactive

Sepharose-bound rabbit muscle phosphorylase subunits and soluble frog muscle and rabbit liver phosphorylase monomers (136). It was found that the inactive immobilized subunit could induce in each case activity in the soluble subunit and it could also transmit its temperature dependence of enzyme activity to the frog muscle subunit but not control properties to the liver subunit. It was suggested that propagation of regulatory properties is more restrictive than induction of activity in phosphorylases even though intersubunit contacts play a role in both cases. Similarly, attempts at making hybrids between matrix-bound subunits of rabbit muscle phosphoglucose isomerase and soluble monomers of other isomerases were carried out to gain information on cooperative interactions between the subunits and to study structural requirements for subunit association (120).

The major advantage of making hybrids on solid-phase is that the method directly yields the desired hybrid and that no separation step is required to obtain the oligomeric form. This can be very difficult when hybridization is carried out in free solution, especially if oligomeric variants do not differ significantly in their electrophoretic or chromatographic properties. Furthermore, to avoid possible artifacts introduced due to the immobilization per se it would also be desirable if the hybrid following construction on solid-phase could be detached from the support and studied in free solution. This would be possible if the hybrid was linked to Sepharose via, for instance, disulfide bonds, provided that the parent protein contains reactive sulfhydryl groups, which can be readily cleaved by addition of a free thiol. This method has been useful in a study of matrix-bound subunits of LDH (123).

An alternative approach would be to bioaffinity-adsorb the native oligomer to an immobilized ligand and after modification of the "free" (not bioaffinity-bound) subunit(s) with an active-site selective reagent elute the hybrid from the affinity adsorbent. This has proven possible in the modification study of bioaffinity-bound HLADH which after reaction with iodoacetic acid could be eluted from the immobilized NADH-analogue (VI). Monoalkylated HLADH was then studied in free solution to investigate whether HLADH follows a flip-flop type mechanism.

A functional advantage of a flip-flop mechanism could be that such an enzyme mechanism results in an increased catalytic efficiency of the enzyme. It has, for example, been argued that strict conformational coupling of molecular events in different subunits (e.g. conformational changes accompanying substrate binding in one subunit may transmit the released binding energy to another subunit

and facilitate product dissociation from that subunit) should lead to an increased rate of substrate turnover per active site compared to an enzyme in which there is no subunit interaction (114,115). Based on such arguments it is also possible to predict that the active subunit of an enzyme molecule that consists of one active and one inactive subunit would be less efficient than each subunit of the corresponding native flip-flop enzyme molecule (since the catalytic flip-flop cycle of the enzyme would be prevented from being completed in the modified enzyme due to the inactive subunit).

The monoalkylated HLADH preparations obtained in this study showed half the activity of the native enzyme when using ethanol or benzaldehyde as substrates (VI). It is known that dialkylated HLADH shows some enzymic activity (94) (when using, for example, benzaldehyde as substrate the activity was found to be roughly 10% of that of native enzyme at pH 8.6 and 26^{o}C (unpublished work)). Therefore, it is possible that the alkylated subunit in monoalkylated HLADH may have some activity. In this situation, if the flip-flop model for subunit interaction is valid for HLADH it is possible that the "slow" subunit in the monoalkylated HLADH would determine the rate of product formation of the hybrid enzyme. This was obviously not found to be the case. However, it could be argued that cooperative subunit interactions are prevented in the monoalkylated enzyme, but it is then unlikely that the hybrid would have half the activity of the native enzyme.

On the other hand, if the monoalkylated HLADH consists of one active and one truly inactive subunit and if subunit reciprocation exists in HLADH one would expect that the subunit turnover number (the steady-state rate divided by the number of active sites per enzyme molecule) would be significantly lower for the monoalkylated HLADH than for the native enzyme (cf. discussion above). However, the turnover numbers of the monoalkylated enzyme and the native HLADH were found to be roughly equal (VI).

From the steady-state kinetic results of the monoalkylated HLADH it is, therefore, possible to conclude firstly that a subunit adjacent to an alkylated monomer is catalytically active, in contradiction with results obtained in a partial alkylation study of HLADH (137), and secondly that the active sites in HLADH function independently of one another and not in a reciprocating manner.

In agreement with this, it has been reported that the transient kinetic time-courses observed for the reduction of aromatic aldehydes are consistent with the ordered ternary-complex mechanism of HLADH (112,138) which does not require

the assumption of subunit kinetic nonequivalence in the enzyme. Thus the biphasic presteady-state kinetic patterns connected with these reactions under single-turnover conditions may just be due to, as previously suggested (111-113), the slow rate of alcohol dissociation from the product ternary complex in comparison with the rate of conversion of the HLADH-NADH-aldehyde ternary complex leading to accumulation of the former complex.

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